Bioremediation of mercury by biofilm forming mercury resistant marine bacteria

Thesis submitted to the
National institute of technology, Rourkela

IN PARTIAL FULFILMENT OF THE REQUIREMENTS
FOR THE DEGREE OF MASTER OF SCIENCE
IN
LIFE SCIENCE

By

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2014

CERTIFICATE

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CERTIFICATE

This is to certify that the project report entitled **"Bioremediation of mercury by biofilm forming mercury resistant marine bacteria"** submitted by **Mr. Edwin Anthony** to the Department of Life Science, National Institute of Technology, Rourkela in partial fulfillment of the requirements for the degree of Masters of Science in **LIFE SCIENCE** is a bonafide record of work carried out by him under my supervision. The contents of this report in full or parts have not been submitted to any other Institute or University for the award of any degree or diploma.

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Acknowledgement

Apart from all my efforts, the success of any project depends largely on the encouragement and precept of many others. I take this opportunity to express my gratitude to the people who have been instrumental in the successful completion of my dissertation. Foremost I would like to give my heartfelt thanks to **Dr. Surajit Das**, **Assistant Professor,** Department of Life Science, National Institute of Technology, Rourkela for his continuous support and motivational words along with his love and care. I feel motivated and encouraged every time I attend his meeting. Without his encouragement and guidance this project would not have materialized.

I also gratefully acknowledge to Dr. Sujit Bhutia, Dr. Rasu Jayabalan, Dr. Suman Jha, Dr.Bibekanand Mallick and Dr. Samir Kr. Patra (HOD), Department of Life Science, National Institute of Technology, Rourkela, for their wholehearted help and cooperation. Furthermore I would like to show my greatest appreciation to Mr. Hirak Ranjan Das and Mr. Subham Basu (PhD Scholars) for their prudent suggestion and valuable advice during my research project. I can't say thank you enough for their tremendous support and help.

Besides all I would like to thank all my lab mates and all other members of LEnME who have willingly shared their precious time during the process of experiments.

Finally, an eminent mention goes to my family and friends for their understandings and, who have supported me throughout the entire process, both by keeping me in good harmony and allowing me for putting the pieces together in course of completing this project.

Last but not the least: I bow my head before almighty for his blessing.

Date: 09-05-2014 **EDWIN ANTHONY**

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Declaration

I hereby declare that the thesis entitled "**Bioremediation of mercury by biofilm forming mercury resistant marine bacteria**", submitted to the Department of Life Science, National Institute of Technology, Rourkela for the partial fulfilment of the Master Degree in Life Science is a faithful record of bonafide and original research work carried out by me under the guidance and supervision of Dr. Surajit Das, Department of Life Science, NIT, Rourkela. No part of this thesis has been submitted by any other research persons or any students.

Date: 09-05-2014 **Edwin Anthony**

Place: Rourkela

LIST OF SYMBOLS AND ABBREVIATIONS USED

gm	Gram
hr	Hour
l	Litre
µl	Micro litre
º	Degree
C	Centigrade
ml	Mili litre
min	Minute
LB	Luria Bertani
MHB	Muller Hinton Broth
MIC	Minimum Inhibitory Concentration
ppm	Parts per million
%	Percentage
MRMB	Mercury resistant marine bacteria
SWNA	Sea water nutrient agar
+	Positive
-	Negative
No.	Number

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Abstract

Predominantly most of the environmental bacteria that were living as a habitant of soil and sediments of lakes and river might embrace the heavy metal genotype to best fitted in the toxic territory. So far *mer* operon mechanism study reveals a lot of facts and data regarding the resistivity secret of mercury resistant bacteria (MRB). Some *mer* operon genes that bacteria harbours were *merA*, *merP*, *mer T*, *merB*, were *merB* responsible for detoxifying organo mercurial compounds by breaking the C-Hg bond. In present study the attempt has been made to mitigate the mercury toxicity using the biofilm forming marine bacteria. There is no doubt that biofilm has always been a sign of spoilage, however from this negativity a beneficial and novel concept was seeking for a human welfare and that we tried to perceive. The EPS present in the biofilm has a specific relation with heavy metals; heavy metals binds with EPS and moves inside the bacteria (bioaccumulation) were it binds with a thiol group and finally a *mer* operon gene coming in to the picture for concluding rest of the activity by detoxifying the toxicity of heavy metal by *mer* operon mechanism. Bacteria are capable enough for these kinds of activity and so they were believe to be a regulator for environmental pollution.

Keywords: Mercury, Bioremediation, Bioaccumulation, Marine Bacteria

1. Introduction

The mysterious world of microbes is unconquerable to even keen to keen eye. Their domain is so design that what goes within that is very tough to perceived, unless the technology comes into the picture. Many micro fauna other than bacteria, fungi, phytoplankton and yeast performs extremely great task to keep themselves settle in their ecosystem. Microbes effort for their survival, growth and reproduction along with the photosynthetic activity and the ability to change the complex substance to simpler one by their unique mechanisms aid the earth ecosystem function and attain as far as a possible dynamic equilibrium.

On the whole, major factor the sunlight is the sole external energy provider for all lives on the earth and all needs of earth too. Array of organisms inhabiting in earth were responsible to produce, consume and recycle other matter in extreme habitat. On the other hand the functional aspect and the stability of the earth are provided by the microbes. If one sees that the ecosystem is under the process of destruction or in adverse condition then the only reason is due to their activities through human and all natural effect. Pollution is always the major problem for the human life and for mighty earth as well, anthropogenic activities claimed to be the greatest problem for the imbalanced ecosystem.

If we see the past, last two millennia or so, the rapid change in the human population and indiscrete uses of Mother Earth’s non-renewable sources were under the edge of declination which ultimately leads to the destruction of habitats. Consequences of such destruction of habitat are shift in human settlement, economic losses, community and species diversity shift, and societal conflict and health effects. Though it is tough for many communities to adjust or tolerate, however biotic community were slot amongst the special case for such tolerance or resistance. In case of industrial realm metals were the concerned element along with organic pollution, bacteria were resistance to metal salt, especially when associated with degrading activities, is of partial significance (Bestetti *et al*., 1996). Tolerance and degradation ability of toxic metal by the bacteria were often addressed separately. Some organic pollutants (fossil fuel or their derivatives, PCBs and TBT among other) often slotted as inorganic ones principally of heavy metals (mercury, cadmium, lead to name a few) in industrial area, which have not taken into account.

Toxic chemical including Hg are let into environment without efficient retention technologies, however some special group of bacteria taking the responsibility to detoxifying/degrading the effect of such toxic chemical (Hg), commonly we called those mercury-resistant bacteria (MRB). These MRB were well growing in the presence of 10 ppm mercury (as $HgCl_2$) in seawater nutrient agar medium (SWNA). Some MRB can grow even in high concentration of Hg (25ppm) and this group were tagged as highly resistance to mercury (Ramaiah *et al*., 2003).

Mercury is one of the chemical element symbolises with Hg and having 80 as its atomic number. Scientific people often known it by ***quicksilver***, earlier it was known as ***hydrargyrum*.** In periodic table it slotted among the d-block element, it is the only element known to be liquid at room temperature, one other element shares the same property is Bromine. Mercury is very rare element in the earth having the occurrence of mass by 0.8ppm. In nature mercury occur in two oxidation states I and II, its higher form (mercury (IV) fluoride) is also been reported but it is of less importance.

1.1. Origin of mercury

Cinnabar is the commonly known mercury ore produced for the human use, rich concentrated with mercury sulphide. As mercury is slotted among the chemical element so it's onerous to create and to destroy too through established chemical or physical means. One can spot mercury in coal and minerals like zinc and copper which considered among the economically important minerals. If it has economical importance than there is no doubt of its frequent use in industry and many other chemical plants, as a result emission or release of mercury to the environment is for sure, resulting in pollution and further to a threat to the globe. The assimilation of mercury into the food chain may worsen matters. As mention earlier mercury always place among the toxic element as it shows a good relation with enzymes and proteins sulfhydryl groups resulting in inactivation of decisive cell function (Strom, 2008).

1.2 General Uses of Mercury

Commonly available products contain mercury or its product:

Medical devices

It was seen that mercury is used in the medical instrument sine very long time. Thermometer, sphygmomanometers, oesophageal dilators were some common medical instrument were we can see the presence of mercury.

Electrical products

Mostly used switches and batteries contains mercury. It was reported that each switch contains around 12 g of elemental mercury. A fancy shoes of kids, lights were used to enhance its look was actually done by these elemental mercury. Mercury is also used in making batteries. The main use of mercury in batteries is to prevent a build up of hydrogen gas that can cause the battery to bulge and leak. Mercury has also been used as an electrode in mercuric oxide batteries.

Measuring device

Mercury has a very unique property of being liquid at room temperature so it expands and contracts evenly with changes in temperature and pressure so it is widely used in many measuring devices.

- ✓ **Barometers** measure atmospheric pressure. (Each may contain 400 g to 620 g of mercury.)

- ✓ **Manometers** measure differences in gas pressure. (Each may contain 30 g to 75 g of mercury.)
- ✓ **Psychrometers** measure humidity. (Each may contain 5 g to 6 g of mercury.)
- ✓ **Flow meters** measure the flow of gas, water, air, and steam.
- ✓ **Hydrometers** measure the specific gravity of liquids.

Pesticides and biocides

Pesticides and biocides were commonly used for the treatment and control purpose and it can be of organic and inorganic mercurial compounds. It can be use as an additive in coating, in hospitals for dressing mean, even for fabrication and laundry use.

1.3 Mercury affecting environment

In spite of the fact that mercury is a natural element, the waste and leftover contamination from erstwhile use of the metal and the under way burning of fossil fuels for energy continue to emit mercury into the environment. Mercury can metamorphose airborne when oil, coal, natural gas or wood, is combusted as fuel or when mercurial litter is incinerated. Once in the air, mercury can be inadequate to the ground with shower of rain and snow. This can then taint soil, bodies of water, and the soul living there. Both metal and organic pollution is of imperative concern for industrial realm, scrutiny in bacterial resistance to metals, especially when analogous with degradative activities, is of practical gravity.

1.4 Forms of mercury in the environment

Mercury prevails in two predominant forms: elemental mercury (Hg^0) and inorganic mercury (Hg^{2+}) (Fig. 1). Elemental mercury is the unblended silvery-white embodiment found in rocks and minerals and more over never seen to be chemically blended. Inorganic mercury can amalgamate with other chemicals to form compounds. Combustion may exempt both elemental and inorganic mercury from materials containing them. Combustion also delivers fine particles that may carry minor amounts of mercury bound to their surfaces. Supplementary elemental and inorganic mercury, other organic mercury compounds for instance methylmercury (MeHg). Subjection to methylmercury is the main cause of public health concern about mercury.

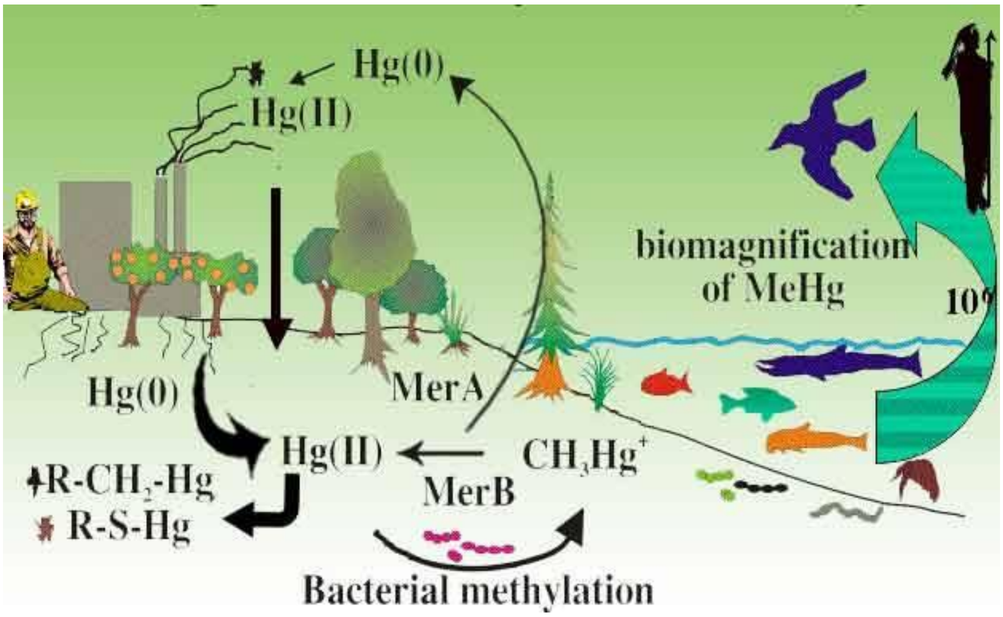


Fig 1. Biogeochemical cycle of Mercury in the environment

1.5 Mercury Pollution

Mercury tainting of the environment by mining activities and industrial has emerged in worldwide contamination of enormous areas of soils and sediments and let to eminent atmospheric mercury levels (Marshall, 1999). Because of lack of suitable agglomerate technologies, efforts to cope with polluted sites are facing the mechanical removal of contaminated material and its expulsion elsewhere. Such treatments are costly and periodically result in remodelled of toxic mercury compounds during the degrading process (Bogdanova *et al*., 1992). Genesis of mercury pollution includes:

- ✓ Production of electrical equipments
- ✓ Chloro-alkali industries

- ✓ Agricultural industries fungicides
- ✓ Coal fired power plants
- ✓ Steel industry

Global scenario for mercury pollution reflects the matter for concern, and this reflection really to be checked out. Asia always coming into the picture by their innovative mind and technical magic's, however something that slots them amongst the bad name is that the pollution contribution to the globe. If we see over the globe, amongst the Asian countries, China is leading with 28% of mercury emission (Wang *et al*., 1987). Other Asian countries were also contributing the same with bit of difference in amount and India is also their within. Population is always a directly proportion to the pollution and the needs of population insist the nation do something offbeat to provide them all necessary need, and to do so industries is the only way of blessing. And now it's well known by all the life that industry means pollution. In one report it was given that the mercury emission by China, India, Japan, and Kazakhstan is around 950Mg respectively (Hao *et al*., 2005). On the other hand deposition of mercury in the North America is significant influence of high level mercury pollution by Asian nation (Feng *et al*., 2009).

In one of the report by U.S. Environmental Protection Agency (EPA), U.S contribute 3% mercury to the mercury pool globally out of which domestic and manmade holds 60% were as 30% by established power plant. Minnesota and northern Wisconsin (Crab lake) known to be the hotspot for mercury pollution in U.S. and this proven by the data that mercury deposition rate is about 7-12.5μg m^{-2} yr (Lamborg *et al*., 2007).

2. Literature review

Mercury and its compounds are disseminating widely all around the earth. Plentiful chemical forms of mercury are toxic to all lives. Nevertheless, bacteria have developed mechanisms of resistance to several of these totally different chemical forms, and thus play a vital role in the global cycling of mercury in the natural environment. Five divergent mechanisms of resistance to mercury compounds have been elucidate, of which resistance to inorganic mercury (HgR) is best deliberate, both in terms of the mechanisms of resistance to mercury as well as resistances to heavy metals in general.

i) Reduced uptake of mercuric ions.

This has been accused in a strain of *Enterobacter aerogenes* where resistance is believed eventual due to the expression of couple of plasmids encoded proteins which cause a reduction in the cellular permeability to Hg^{2+} ions (Phung, 1996).

ii) Demethylation of methylmercury followed by conversion to mercuric sulphide compounds.

In *Clostridium cochlearium* T-2P two plasmids encoded genetic factors are assume to be responsible for the demethylation of organomercurial compounds which are eventually inactivated by reaction with hydrogen sulphide to form insoluble mercuric sulphide (Silver, 1996).

iii) Sequestration of methylmercury.

Continuous production of hydrogen sulphide is a source for maintaining methylmercury at sub-toxic level in *Desulfovibrio desulfuricans*, from the dissimilative diminution of sulphate, so that it can react with methymercury to form indissoluble dimethylmercury sulphide (Baldi, 1994).

iv) Mercury methylation.

Notwithstanding the fact that methymercury is predominantly considered to be more toxic than Hg^{2+}, in some bacteria methylmercury characterises to be less toxic form, perhaps due to successive volatilisation from the cell. Methylation has been recognized in bacteria from water, soil, sediment and the gastrointestinal tract, and is both plasmid and chromosomally encoded (Barkay *et al.* 2003 and Miller *et al.*, 2005). In *Desulfovibrio desulfuricans* the methylation of mercury exist as a duo step process which elaborates the transfer of a methyl group from methyltetrahydrofolate to methylcobalamin to Hg.

v) Enzymatic Reduction of Hg^{2+} to Hg^{0}

Reduction seen both in Gram-negative and Gram-positive aerobic bacteria from a variety of natural and clinical environments across the globe, and as such has become the best studied of the mercury resistance mechanisms. Mercury resistance is often located on conjugative plasmids and/or transposons (Nascimento 1990) and in particular is often borne on class II transposable elements, typified by that carried by Tn2*I*. Furthermore, such HgR plasmids or transposons often carry resistances to other heavy metals and/or antibiotics.

2.1. Bacterial bioremediation of mercury contamination

2.1.1. Mercury in the environment

The only metal in liquid form which we commonly known is mercury, it also exists as gas too because of its high vapour pressure and also categorise the most toxic amongst the heavy metal (Marshall, 1999). Mercury also rated the sixth most toxic chemical amongst the list of hazardous compound. Mercury also available in the form of mineral (cinnabar-HG-S), as mercuric oxide, sulphate mineral, oxychloride (Bloom *et al*., 1997) or also as elemental mercury by the contribution of volcanic eruption. Mercury globally dispersed a lot and because of which it emits lot of physiochemical deviation in biogeochemical cycle (Fig 2). Some natural processes that responsible for the Hg emission are:

a. Degassing from geological mineral deposits,

b. Volcanic eruption,

c. Formation of divalent mercury in the aquatic system by the process of photo reduction and

d. Formation of elemental and methyl mercury by biological process.

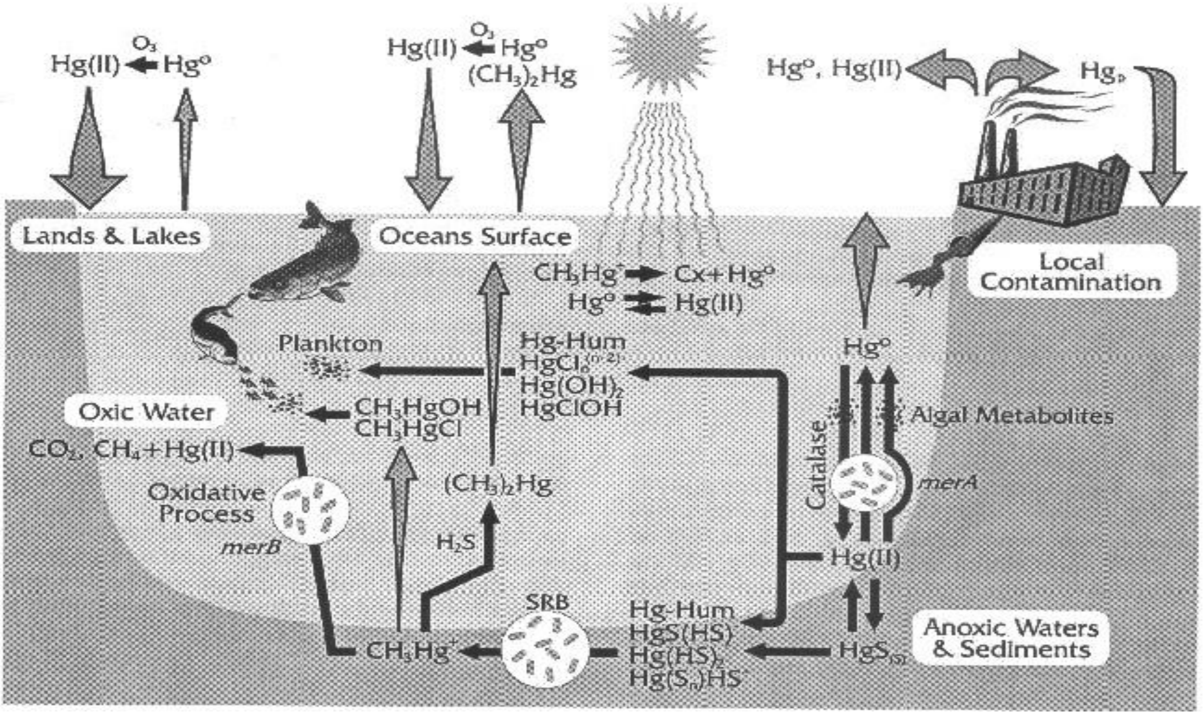


Fig 2. Fate of Mercury in the environment

As we all know mercury undisputedly occurs naturally and toxic concentration in many environmental sites, emission of mercury caused by anthropogenic activities (mainly through chlorine production and alkali electrolysis), combustion of fossil fuel, or fossil fuel incineration, all these undoubtedly contributing to the mercury pool and participating in the biogeochemical cycle. Mercury possess a diverse properties like liquid at room temperature, odourless, tolerate wide range of temperature and most important it’s highly volatilization nature and just because of this mercury always the first choice by the industry peoples.

Recently it was reported that the mercury contamination or emission is becoming a great threat for the people worldwide and for environment too (Lopez *et al*., 2009). In aquatic world, mercury concentration ranges: Open Ocean (0.5-3 ng 1^{-1}), river and lakes (1-3 ng 1^{-1}) and costal seawater (2-15ng 1^{-1}) (El-Agroudy, 1999).

People indirectly consuming mercury (mostly as methyl mercury) while having fishes or any aquatic food stuff in their dinning. Actually via food chain process the mercury accumulated by carnivorous fish by the process of biomagnifications. Minamata is one of the lethal disease caused by consuming Hg, this was first reported in Japan in late 1950, and over 3000 people were affected by effluents released by chemical manufacturing plant in to Minamata Bay.

2.2 Biochemical Basis and Molecular Basis of Bacterial Mercury Resistance:

a. Formation of insoluble HgS:

In the presence of hydrogen sulfide, mercuric ions (Hg^{2+}) spontaneously precipitate as mercuric sulfide (HgS) (Jernelov, 1979). Under anaerobic conditions, the formation of mercuric sulfide effectively reduced availability of mercuric ion for biological conversions. In the presence of oxygen, mercuric sulphide may be converted to methyl mercury by bacteria; however, this occurs at a rate 100- 1000 times slower than mercuric ion methylation. Therefore, the presence of sulfide reducing bacteria prevents methyl mercury [$(CH3)_2Hg$] and mercuric sulfide in the presence of hydrogen sulfide. Mercuric ion may also be reduced to the volatile elemental mercury by resistant bacteria. This reaction results in the release of mercury from aquatic systems (Jernelov, 1972). Mercury volatilization might be expected to occur readily than methylation due to the large numbers of bacteria capable of carrying out this reaction in aquatic sediments and the kinetics of volatilization in bacterial cultures compared to methylation.

b. Enzymatic reduction Hg^{2+} to Hg^0 and volatilization:

The biochemical basis of resistance to inorganic mercury compounds such as $HgCl_2$ appears to be quite similar in several different species. It involves the reduction of Hg^{2+} to volatile Hg^0 by an inducible enzyme, mercuric reductase. This reductase is a flavoprotein, which catalyzes the NADPH-dependent reduction of Hg^{2+} to Hg^0. Since mercury has such a high vapour pressure, it volatilizes and the bacterial environment is left mercury free. This mercuric reductase is found intracellularly (Schottel *et al*., 1985).

As a response to toxic mercury compounds globally distributed by geological and anthropogenic activities, microorganisms have developed a surprising array of resistance systems to overcome the poisonous environment. An extensively studied resistance system, based on clustered genes in an operon (*mer* operon), allows bacteria to detoxify Hg^{2+} into volatile metallic mercury by enzymatic reduction (Hong *et al*., 1993). Mercury-resistance determinants have been found in a wide range of Gram-negative and Gram-positive bacteria isolated from different environments. They vary in the number and identity of genes involved and are encoded by *mer* operons, usually located on plasmids and chromosomes (Wang *et al*., 1987); they are often components of transposons and integrons (Liebert *et al*., 1999). Two main *mer* determinant types have been described: narrow-spectrum *mer* determinants confer resistance to inorganic mercury salts only, whereas broad-spectrum *mer* determinants confer resistance to organomercurials such as methyl mercury and phenyl mercury, as well as to inorganic mercury salts. The functions of *mer* operon are as follows:

i. Transport of Hg^{2+} into the cell

ii. Enzymatic NADPH dependent conversion of the ionic mercury into relatively less toxic elemental mercury (Hg^0)

iii. Regulation of the functional genes

iv. Cleavage of mercury from the organic residue and the resistance is termed as “Broad spectrum”

The genes involved in *mer* operon are (Fig. 3)**:**

a) *merT*, *merP* (Transport)

b) *merA* (Mercury reduction)

c) *merB* (Cleavage of mercury from organic residue)

d) *merR* and *merD* (regulation)

e) *merC* and *merF* (Membrane proteins, conferring transport functions)

f) *merG* (resistance to phenyl mercury)

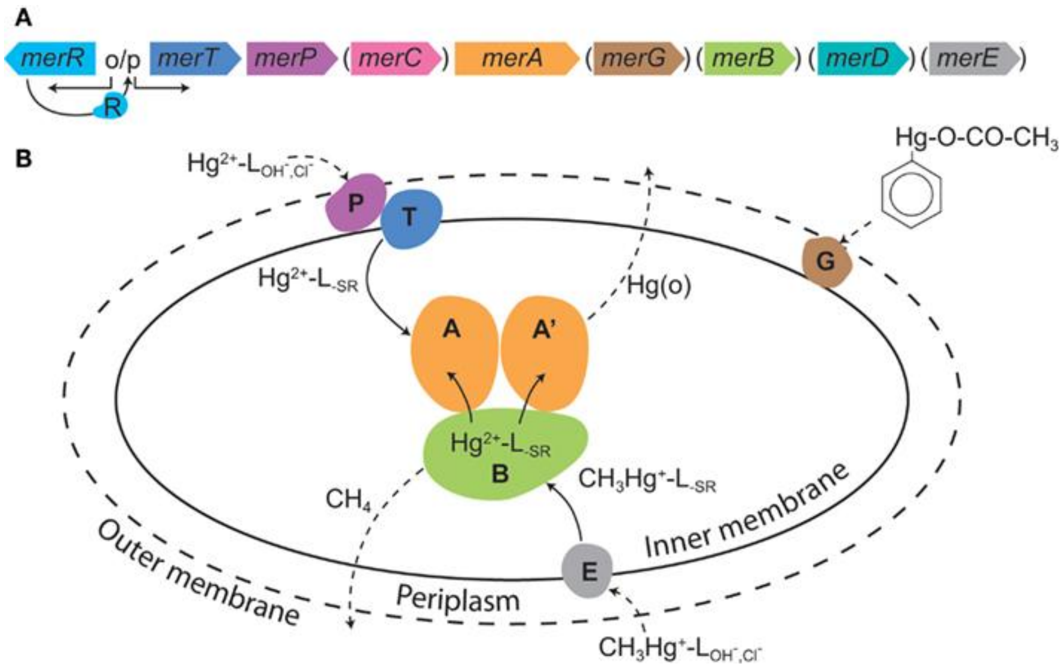


Fig. 3 The *mer* operon including the regulators (*merR* and *merD*), transporters (*merP* and *merT*), mercuric reductase (*merA*) and organomercurial lyase (*merB*).

Different mer genes in mer operon play different roles. The functions of these genes are as follows:

1. ***merR*:** Metalloregulatory DNA binding protein that acts as a repressor of both its own and structural gene transcription in the absence of Hg (II). In addition it acts as a positive effector of structural gene transcription when Hg (II) is present.

2. ***merB*:** Organomercury lyase, catalyzes the protonolytic fragmentation of organomercurials to the parent hydrocarbon and Hg(II) by SE2 mechanism.

3. ***merA*:** Mercuric ion reductase, is an FAD containing and redox active disulfide containing enzyme with homology to glutathione reductase. This enzyme reduces Hg^{2+} compounds to the metallic mercury Hg^{0} which is obviously less toxic to them (Mirzaei, 2008). It has the unique capacity to reduce Hg (II) to Hg (0) and thereby complete the detoxification scheme. Based on a comparison with other bacterial periplasmic binding, protein-dependent transport systems, it has been proposed that Hg^{2+} diffuses across the outer membrane (Barkay and Selifonova, 1994).Mercuric ions are transported outside the cell by a series of transporter proteins. This mechanism involves the binding of Hg^{2+} by a pair of cysteine residues on the *merP* protein located in the periplasm Hg^{2+} is then transferred to a pair of cysteine residues on *merT*, a cytoplasmic membrane protein, and finally to a cysteine pair at the active site of *MerA* (mercuric reductase) (Landale *et al*., 1992). Next, Hg^{2+} is reduced to Hg^{0} in a NADPH dependent reaction. The non-toxic Hg^{0} is then released into the cytoplasm and volatilizes from the cell.

4. ***mer*D:** A small, cysteine-rich open reading frame (ORF) lying just beyond the *mer*A gene of Tn501. Purified *mer*D binds to mer O although with a lower apparent affinity compared to *mer*R. Thus, *mer*D appears to be an antagonist of *mer* R function, perhaps replacing it at *mer*O although other mechanisms or roles for *mer*D have not been ruled out. *Mer*D is also unique protein with no homologs with identified functions.

5. ***mer*P:** Although *mer*P does not resemble any periplasmic transporter involved in normal metabolism, the *merP* motifs appears to be quite ancient and widely disseminated in proteins involved in both membrane transiting and cell interior trafficking of thiophilic metal cations. Interstingly, *mer* P is not essential for Hg (II) uptake as *mer* T alone will suffice.

6. ***mer*T:** *mer* T is the other player in Hg (II) transport in both Gram-positive and Gramnegative bacteria (excepting *Acidothiobacilli* which apparently use only *mer* C) (Miller *et al*., 2003). There are no reported physical studies on *mer* T, largely owing to the difficulty of such studies on membrane proteins. Possible homologs of the *mer* inner membrane proteins with known functions have not been spotted, although doing so might be difficult.

7. ***mer*C:** This 161-residue membrane-bound protein with four foresee transmembrane helices is consider the largest amongst the small mer operon encoded membrane proteins. Its occurrence in only one of the first two, otherwise very similar *mer* operons sequenced was the first hint of the mosaic character of the operon (Miller *et al*., 2003). Studies concluded that *mer* C is evolving differently than genes immediately adjacent to it in the operons where it occurs and may be also evolving in different hosts. It has been suggested that *mer* C may be needed under conditions of very high Hg (II) exposure (Nies, 1999), but this point has not been explicitly tested. *mer*C is not uniquely associated with *mer* B or *mer* G.

8. ***mer*F:** The *mer* F gene was first noted between the *mer*P and *mer*A genes in a plasmid-borne *mer* operon in an environmental pseudomonad (Bruce *et al*., 1997). Nigel Brown's group manifest that *mer* F is sited in a crude membrane fraction procure from radio labelled maxicells. Expression of *mer* F smooth the way for volatilization of Hg (II) however this activity was not intensified by *mer* P.

2.3. Bacteria resistance to mercury:

Industrialization, one of the responsible element for the distribution of toxic mercury globally along with anthropogenic and geological activities, however surprisingly microbes develops the array of resistance mechanism to deal with such kind of toxic element, especially mercury toxicity. A unique resistance system employed within bacteria in the clustered gene in an operon (i.e. *mer*), by which bacteria enable to detoxify Hg^{2+} into volatile mercury by the efficient action of enzymatic reduction (Bruce *et al*., 1997). The examined reports stated that Gram-negative and Gram-positive possess a wide range of Mercury-resistance decisive factor when isolates taken from the different location. Unique genes were encoded by the *mer* operon which located on the plasmid, chromosome, transposons and integrons; however genes were varying in number and their identity.

Organic as well as inorganic ligands were present within mercuric mercury complexes, these ligands own the property to absorb to the variable particulates and also they have the well defined system which has the affinity and reactivity to thiol group (Barkay and Wagner-Dobler 2005; Miller *et al.*, 2005). So to examine the resistance level of mercury, mercury bioavailability plays a crucial role.

Basically three major functions comprises by narrow mercury resistance operon (*mer*): transportation of Hg^{2+} into the cell, conversion of ionic mercury into less toxic elemental mercury (Hg^0) by enzymatic NADPH-dependent conversion and mechanism to regulate functional gene. Now if we consider detoxification of organic mercury, mercury should be cleaved off from the organic residue and for that specific genes were employed by the system which designated as *mer*T, *mer*P (for transport), *mer*A (for reduction of mercury), *mer*B (for cleavage of Hg from organic residue), *mer*R and *mer*D (for regulation). However some other *mer* genes were also been identified recently which were known to be membrane protein; *mer*F and *mer*C . In the same junction of recent identification merG is known to grant resistance to phenyl mercury (Pan-Hou, 1999). To activate the expression of resistance, most of the mercury resistance operon needs to be get induced (Misra, 1984). Mercury resistant bacteria are always inducible and never constitute for Hg-reductase activity, these phrase were given by many researcher. On the other hand *mer*A induced when the concentration exceeds 50 ppm (Morel 1998 and Kraepiel *et al*., 1998).

2.4 Mercury-resistance bacteria to bioremediation

Effluent from the industry into the water source is the major concerned for water pollution. Effluent contains lots of hazardous stuff along with some amount of mercury too; to deal with the Hg, bacterial community is the best one which coming into the picture, they got the unique ability to detoxify mercury and for that only it can be utilize to bio remediate mercury contaminated water. The way literature praise the microbial effort for the detoxification of mercury it seems as if this potential was solely restricted to microbial community. Nevertheless, plants were also standing tall enough to detoxify mercury by transforming organic and inorganic mercury to elemental mercury by the process of phyto-remediation (Senecoff *et al*., 1998). One of such plant is yellow poplar, its transgenic version carry the potential for dealing with mercury contamination area. On the other hand, stout-tree root act as a medium by providing niches for the inhabitation of mercury reducing bacteria by stabilizing the soil surface. As in every merits some lacunas seeking outward, so with these plant community also in detoxifying Hg, during detoxification of mercury, volatile Hg^0 emits out and on global scale it is non-satisfactory stuff which may be the agent for enhancing mercury pool.

Bacteria no doubt efficiently detoxify the mercury by reducing its valance charge but the problem is elemental mercury which is no less toxic to many lives. So to overcome with this problem, initial effort were made to retain the mercury in the bacterial bioreactor by trapping the elemental mercury in the form of globule up to 5µm using genetically engineered mercury-reducing bacteria.

3. Objectives and Work plan

Taken into account for above mention conceptions, present research is based on the following objectives:

3.1 Objectives

- ✓ Isolation and individualization of mercury resistant bacteria.
- ✓ To study bio-availability of absorbed mercury in EPS.
- ✓ To study the tolerance level and mechanism of mercury resistant in the isolates.
- ✓ To study the expression of *mer* A in biofilm under various condition

3.2 Work plan

10 isolates

⇩

Minimal inhibitory concentration (MIC) test

⇩

Biofilm screening and quantification

⇩

***mer*A gene amplification**

⇩

Extraction of EPS from bacteria grown under various concentration of Hg stress

⇩

FTIR analysis of extracted EPS at an interval of 24 h, 48 h, 72 h and 96 h.

⇩

Expression of *mer*A gene

4. Methods and Materials

4.1 Sample collection and isolates

Both water and sediment samples were collected from Bay of Bengal along Odisha and mercury resistant marine bacteria were isolated from the samples using standard procedure (Ramaiah, 2007). The study sites include Bhitarkanika (20°44.33'N & 086°52.06'E), Chilika (19°44.582' N & 85°12.768'E), Gopalpur (19°19.218'N & 084° 57.730'E), Paradeep (20° 55.44' N & 86°34.62' E) and Rushikulya (19°22.647'N & 85°03.165'E). The pure cultures of bacteria were previously isolated and stored in the laboratory. Bacterial cultures were retrieved and analysed further using their pure culture. The isolates used during this study include BW-03, CS-605, CW-603, RW-402, GW-502, PW-04, GW-702, PW-401, PW-802, CW-102 RW-406, RW-203, RW-401, CS-205, RW-101, CW-304, CW-503, PW-702, PW-216, and CS-09.

4.2 Minimal Inhibitory Concentration (MIC) test

Minimal inhibitory concentration is best defined as the completely prevention of bacterial growth in modest concentration of metal. MIC was determined by using micro-broth dilution technique in a micro-titre plate (CLSI, 2006). The protocol used has been described briefly below.

The foremost task is to mark/labelled the well on 96-well plate to avoid the confusions.

1. Basically 12 columns were present in the standard 96-well plate, 300 µl of MHB (+100 ppm of $HgCl_2$) was added in 1st column and 150 µl of MHB (+100 ppm of $HgCl_2$) in the last column.
2. Remaining wells were filled with 150 µl of sterilised MHB.
3. Serial dilution was did were 150 µl form 1st well is taken and transferred to next well and subsequently till 10th well.
4. 150 µl of content was discarded from10th well.

5. 20 µl of McFarland culture was added to the entire well except 12th column wells which acts as a negative control.
6. After all this the plate was incubated at 37°C for 24 h.
7. Finally, after 24 h absorbance was taken at 595 nm in the ELISA plate reader.
8. According to the negative control the MIC was determined after carefully inspection of each well at OD_{595}.

4.3 Biofilm screening and quantification

As samples were collected from different study sites so we have to know which sample can be further used for our research purpose, so screening is one of the most important parts of this study. Different methods were there to screen the sample; however we adopted two basic methods or assay, namely; Glass tube assay and micro-titre plate assay (Jain et al., 2014).

A. Glass tube assay

1. In each tube 1ml of LB (Luria Bertani broth) were taken and sterilised by autoclaving it.
2. 100 µl overnight shaking culture was taken in to 1 ml of LB tube.
3. Static incubation is done for 48 h at 37°C.
4. After incubation, the media was discarded or decant.
5. The tube was gently washed with either PBS or with distilled water.
6. After 5 min of washing, 1 ml of 0.2% aq. solution of crystal violet was added to the tube.
7. Again the tubes were washed as same done in after incubation.
8. After around 15-20 min the tubes were washed with 95% ethanol.
9. Finally, we can see the ring formed on the wall of tube.

10. The distinct ring indicates the good biofilm forming strain, we can keep the record by using "+" mark, the more "+" mark the good biofilm producing strain was.

B. Micro-titre plate assay

1. Isolates from fresh Nutrient agar plate inoculated in LB broth for 24 h at 37°C in static condition.
2. Dilute overnight culture 100 times and fill 200 µl aliquot into each well of a 96well plate/ micro-titre plate.
3. The plate was incubated at 24 h, 48 h and 72 h respectively at 37°C.
4. After incubation, the plate was washed with PBS to remove the free floating bacteria.
5. The wells were stained with 0.1% w/v crystal violet for 1 min.
6. Excess stain was washed with distilled water.
7. Further, the plate was added by 95% ethanol and plate was kept for drying after removing the ethanol and absorbance was taken at 595nm in the ELISA plate reader.

4.4 *merA* gene amplification

4.4.1. Preparation of bacterial lysate

Before proceeding towards *merA* amplification we prepared a bacterial lysate (Boiling lysis). Assorted steps were there to conceive a bacterial lysate, those were:

- 200 µl of bacterial culture was taken in 1.5 ml MCT.
- Spin at 6000 rpm for 5 min.
- Discard the supernatant
- Add and re-suspend in 300 ml milli Q water
- Keep in water bath at 100°C for 10 min
- Immediately keep on ice for 5 min
- Finally, spin at 10000 rpm for 10 min
- Transfer the supernatant to a fresh MCT

4.4.2. Amplification of conserved regions of merA

Following primer were used for the amplification of *merA* gene: F1merA-5' TCGTGATGTTCGACCGCT3'; F2 merA-5' TACTCCCGCCGTTTCCAAT3' (Sotero-Martins *et al*., 2008). The total volume was 25µl for the amplification reaction; the reaction was performed by using thermal cycler (BioRad). To perform this experiment we prepared a PCR mixture were of; 11.4 µl of Milli Q, 2.5 µl of 1X enzyme buffer, 1.5 µl of $MgCl_{2,}$ 0.6 µl of dNTPs, 2.0 µl of *merA* F, 2.0 µl of *merA* R, 11.4 µl of Taq Pol and 4.0 µl of Template .

Amplification condition was optimized as (Fig .4);

- Pre denaturation step at 94°C for 1 min
- 30 cycles of 94°C for 1 min, 55°C for 1 min
- Extension step at 72°C for 1 min
- Final extension at 72°C for 7 min.

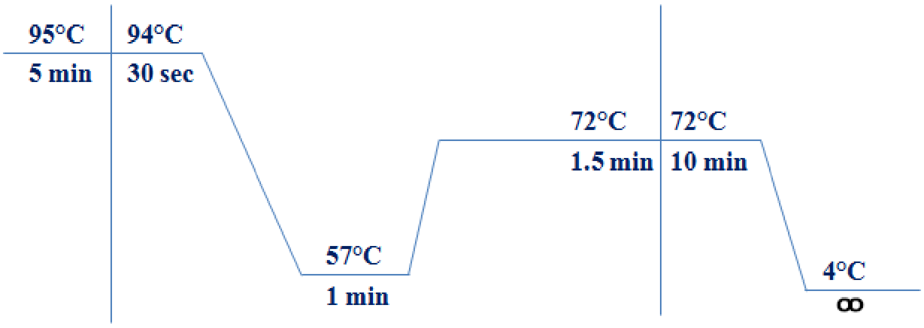


Fig 4. Amplification condition for *merA* amplification

The PCR products were examined using agarose gel electrophoresis (1.5% agar) and visualized under UV light in Gel Documentation System (Bio-Rad). A mercury resistant strain of *B. thuringiensis* PW-05 was used as the positive control.

4.5 Extraction of EPS from bacteria grown under various concentration of Hg stress

Extraction of EPS was done both in the presence of Hg and in absence of Hg, so that a comparison can be made after the FTIR analysis. There were several steps for the extraction of EPS.

1. 100 ml of LB broth of PW-702 was prepared in 250 ml of conical flask.
2. Incubated for 24 h, 48 h, 72 h, and 96 h at 37°C.
3. After incubation the culture was taken into 50 ml of two falcon tubes.
4. Tubes were taken for centrifugation at 6900 rpm for 30 min at 4°C.
5. The supernatant which forms were taken into another 50 ml of four falcon tubes, the supernatant was measured around 160 ml so in each falcon tube 20 ml of supernatant was taken.
6. Equal amount of chilled ethanol was added to those four falcon tube containing supernatant and kept at 4°C for overnight.
7. After overnight, four falcon tubes were taken for centrifugation at 6900 rpm for 30 min at 4°C.
8. The pellet was collected and supernatant was discarded, this obtained pellet is EPS.
9. Finally, pellets were kept in desiccators to remove moisture.

4.6 FTIR analysis of extracted EPS of different time interval

In our study FTIR analysis is done basically for examine the binding of Hg with thiol group. There were several stretches at different wave number (Table 1).

Table 1. Functional groups with correspond to their wave number

Functional Group Stretch	Wave Number cm^{-1}
S-H Stretch	2339-2349
C-S Stretch	710-570
C=S Stretch	1275-1030
S-S Stretch	700-550
S=O Stretch	1225-980

After the FTIR analysis, we got some peak and by that we can interpret that at which stretch Hg binds to the EPS.

4.7 Relative expression of *merA* gene with respect to time interval and mercury stress

Before moving for examine the expression on *merA* we did RNA isolation and cDNA synthesis of the specific strain.

4.7.1 RNA Isolation:

1. Overnight culture was taken in 1.5 ml MCT and centrifuged it for 5 min at 1.3 g.
2. Supernatant was discarded and 0.5 ml of Trizol was added in the pellet and kept it for 5 min at room temperature.
3. 0.2 ml of chloroform was added and vigorously shakes for 15 sec and kept it for 2-5 min at room temperature.
4. The mixture was centrifuged at 12000 rpm for 15 min at 4°C.
5. Aqueous phase obtained after centrifugation was collected in the fresh MCT.
6. 0.5 ml of isopropanol was added and mixes properly.
7. Again a centrifugation for 12000 rpm for 10 min at 4°C.
8. The supernatant was removed and washed with 1 ml of 75% ethanol and centrifuged at 7500 rpm for 5 min at 4°C.
9. After centrifugation the ethanol was discarded and the RNA pellet inside it left for 10 min for air drying.
10. Finally, 40 µl of DPEC water was added and the concentration was checked.
11. Concentration of extracted RNA was examined using Nano drop (Table 2).

Table 2. RNA concentration, with and w/o Hg at different time interval

Sl. No	Time Interval	Concentration (ng/µl)	260/280	260/230	A230	A260	A280	A340
1	24 h with Hg	112.8	1.24	0.19	0.293	0.56	0.46	0.0
2	24 h w/o Hg	103.9	1.31	0.24	0.21	0.52	0.040	0.010
3	48 h with Hg	44	1.4	0.2	0.112	0.22	0.116	0.0
4	48 h w/o Hg	37	1.30	0.24	0.078	0.018	0.014	0.0
5	72 h with Hg	248.8	1.57	0.28	0.069	0.079	0.007	0.0
6	72 h w/o Hg	63.2	1.46	0.21	0.149	0.032	0.022	0.0
7	96 h with Hg	35.1	1.71	0.31	0.138	0.018	0.010	0.0
8	96 h w/o Hg	25.6	2.56	0.19	0.66	0.013	0.05	0.0

4.7.2 cDNA Synthesis:

DNase treatment is the primary step of cDNA synthesis, were the removal of genomic DNA from the RNA was carried out.

1. A mixture was prepared were; 5 µl of RNA, 1 µl of DNase, 1 µl of buffer and 3 µl of DEPC water was taken.
2. Mixture was allowed for incubation for 30 min at room temperature.
3. 1 µl of 50 mM EDTA was added to it.
4. Again the mixture was left for 10 min of incubation at 65°C.
5. Prepared RNA was used as a template for reverse transcriptase.
6. Reverse transcription reaction mixture was prepared were 4 µl of reaction buffer, 1 µl of RNase inhibitor, 2 µl 10 mM dNTPs and 1 µl of Reverse Transcriptase was taken and examine the concentration of cDNA using Nano drop (Fig 5) and (Table 3).

Table 3. cDNA concentration, with and w/o Hg at different time interval

Sl.No	Time interval	Concentration (ng/µl)	260/280	260/230	A230	A260	A280	A340
1	24 h with Hg	1330.0	1.56	1.87	0.298	0.556	0.357	0.00
2	24 h w/o Hg	1568.2	1.56	1.50	0.418	0.627	0.403	0.00
3	48 h with Hg	1562.7	1.42	1.69	0.370	0.625	0.397	0.04
4	48 h w/o Hg	1268.5	1.58	1.68	0.303	0.507	0.331	0.00
5	72 h with Hg	2433.3	1.32	1.51	0.642	0.973	0.735	0.01
6	72 h w/o Hg	2047.5	1.51	1.83	0.448	0.819	0.542	0.02
7	96 h with Hg	3388.0	1.57	1.97	0.688	1.355	0.861	0.01
8	96 h w/o Hg	2147.0	1.47	1.85	0.465	0.859	0.584	0.00

RT program was set as Fig 5:

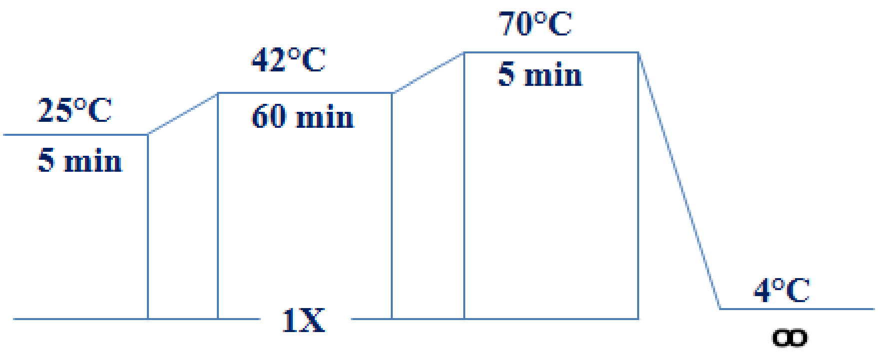


Fig 5. RT program for cDNA synthesis

4.7.3 Real Time PCR for the expression of *merA* gene

RT PCR used to examine expression profile of binding of Hg to EPS at different time interval; this data will provide the detailed structure of binding affinity towards EPS. cDNA was diluted to the concentration of 200 ng/µl.

Reaction mixture of RT PCR is depending upon the concentration of cDNA, so it is very important to dilute the cDNA very carefully. For reaction mixture 112.5 µl of Syber green, Reverse and Forward primer 10 µl, Milli Q 68.5 µl and DNA Template 54 µl was taken, were the final volume is 255 µl. One house keeping 16s rRNA along with our *merA* gene was taken for RT PCR with equal volume and concentration, and following PCR program was set. (Fig 6)

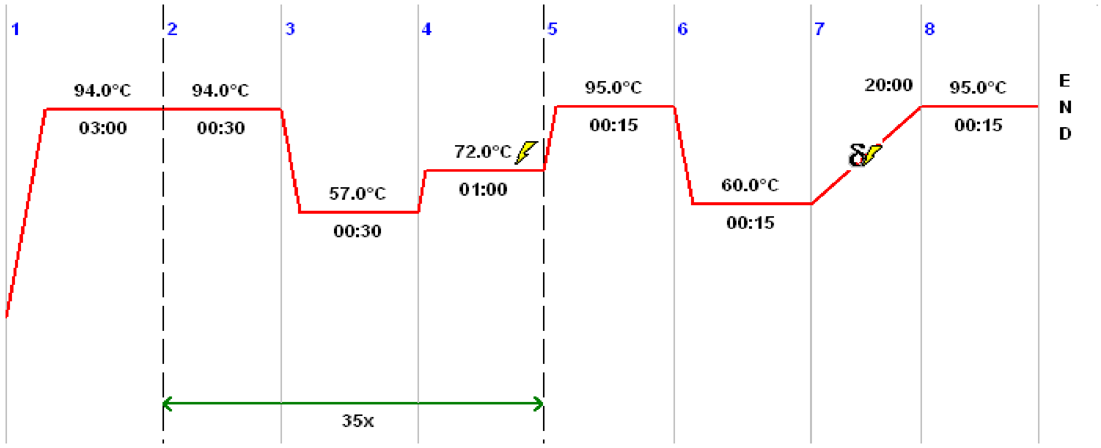


Fig 6. PCR program for expression of *merA* gene

5. Results

5.1 Isolation of mercury resistant marine bacteria (MRMB)

A whole set of 20 bacterial strains were showing obvious distinct colony morphology and found to be well proficient of tolerating Hg when the set of strains were grown on SWNA +10ppm $HgCl_2$ plates. Further the strains were taken for the resistance mechanism toward the Hg by several test and results were given below.

5.2 Phenotypic characterization of the isolated colonies

The colonies were observed and according to their shape and colour a set of collective observation were displayed (Table 4) and (Fig 7).

Table 4. Colony morphology of the isolated strain

Sl. No	Strain Name	Colour Shape
1	BW03	Yellowish Round
2	CS605	White Very small round
3	CW 603	Yellowish Round
4	GW 502	White Very small round
5	RW 402	Yellowish Round
6	PW04	White Round
7	PW401	White round
8	GW 702	White Very small round
9	PW02	Whitish yellow round
10	CW102	Yellowish Round

Sl. No	Strain Name	Colour Shape
1	RW 401	White Very small round
2	BS 202T	White Very small round
3	CW 304	Creamish Round
4	RW 406	Whitish Round
5	CW 503	Yellowish Round
6	RW 203	Yellowish Small
7	PW 702	White round
8	PW 216	White Very small round
9	CS 205	Clear White Round very small
10	CS 09	Whitish Round

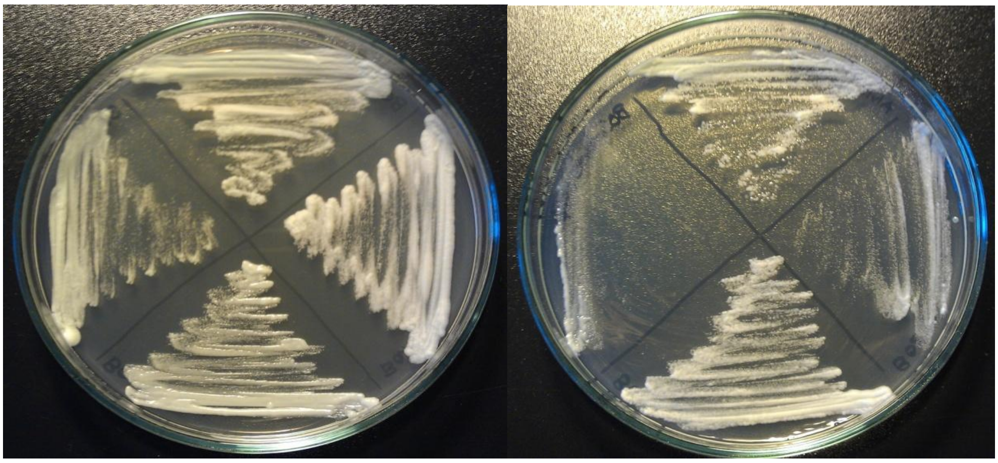


Fig 7. Isolated colonies on SWNA +10 ppm $HgCl_2$ plates

5.3 Minimal Inhibitory Concentration Determination

Minimum inhibitory concentration is the lowest concentration of metal that completely prevented bacterium growth (Ramaiah, 2007). MIC of all 20 strains was listed below (Table 5) and (Fig. 8 & 9).

Table 5. MIC values of Bacterial isolates

Sl no	Strain	MIC (ppm)
1	BW03	12
2	CS605	6.25
3	CW 603	25
4	GW 502	24
5	RW 402	12
6	PW04	1.56
7	PW401	50
8	GW 702	12
9	PW802	50
10	CW102	1.56

SlNo.	Strain	MIC (ppm)
1	PW 702	6.25
2	CS 09	1.56
3	CS 205	6.25
4	PW 216	50
5	BS 202T	0.39
6	CW 503	0.39
7	RW 203	1.56
8	CW 304	0.78
9	RW 406	25
10	RW 401	25

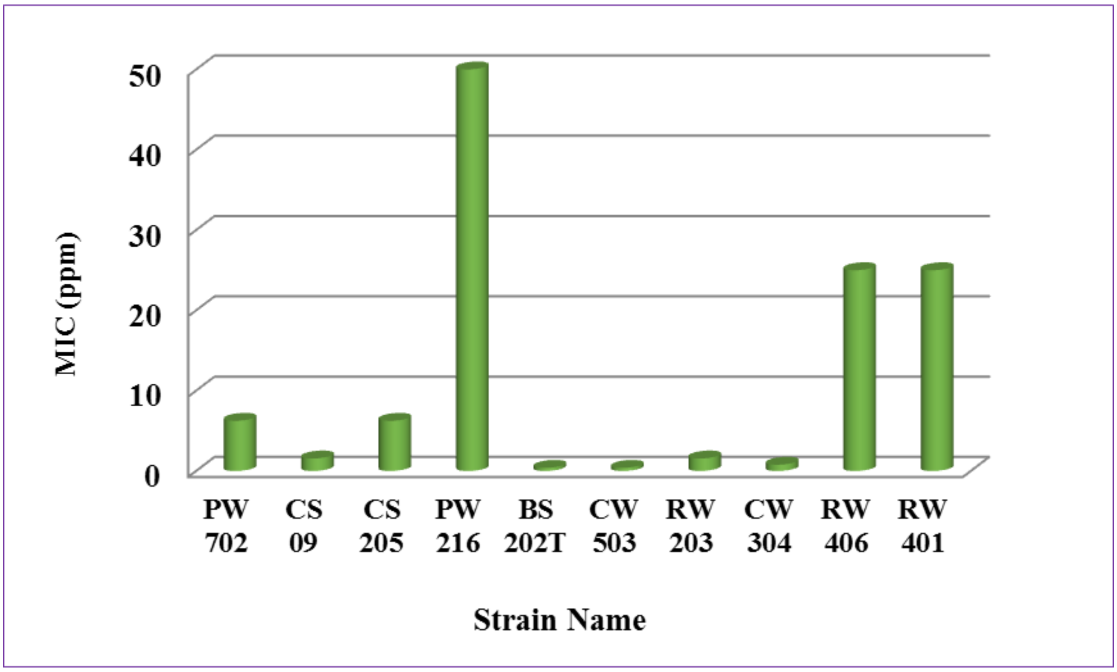


Fig 8. Graphical representation of MIC results of Bacterial isolates

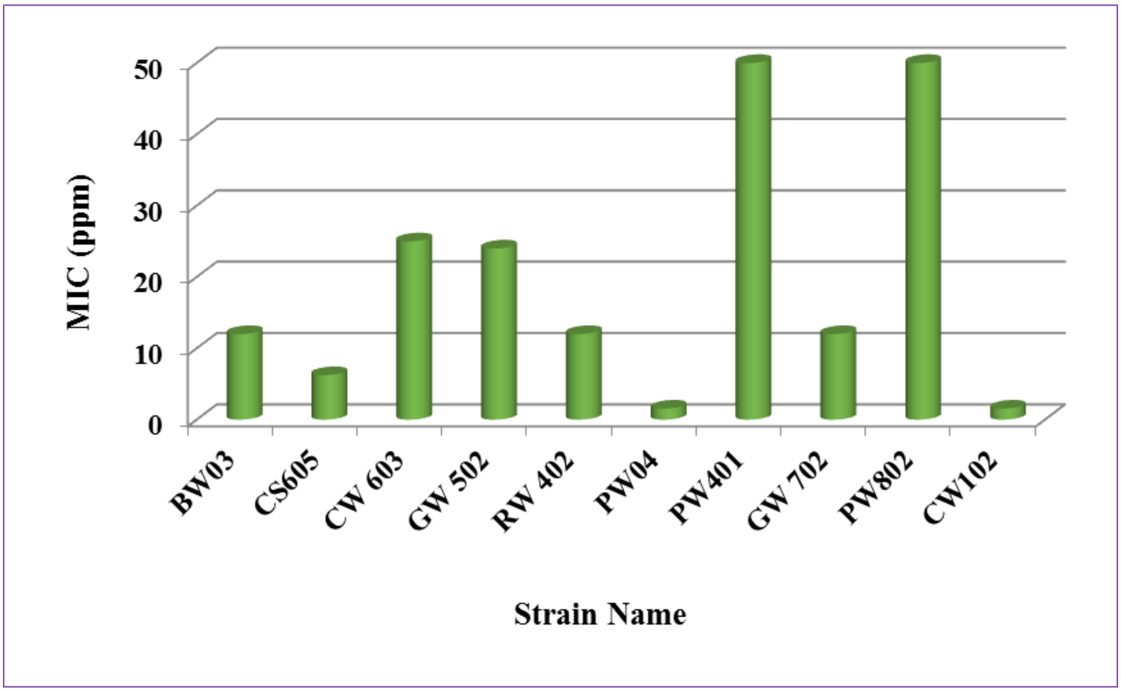


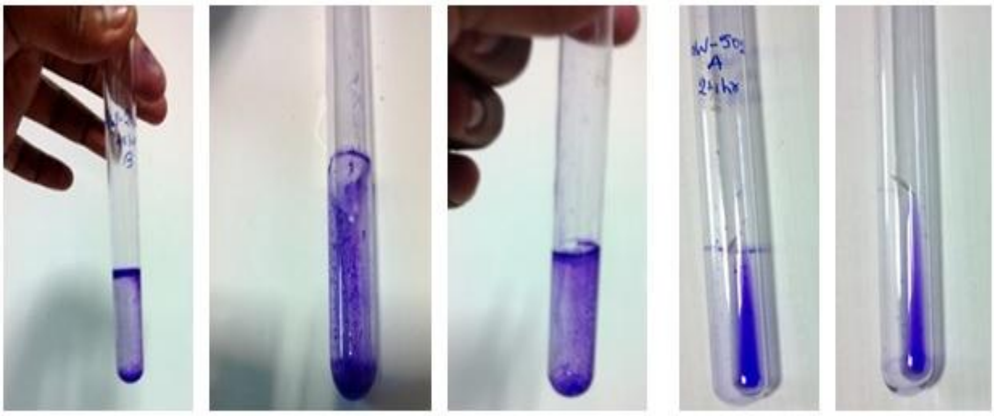
Fig 9. Graphical representation of MIC results of Bacterial isolates

5.4 Biofilm Screening

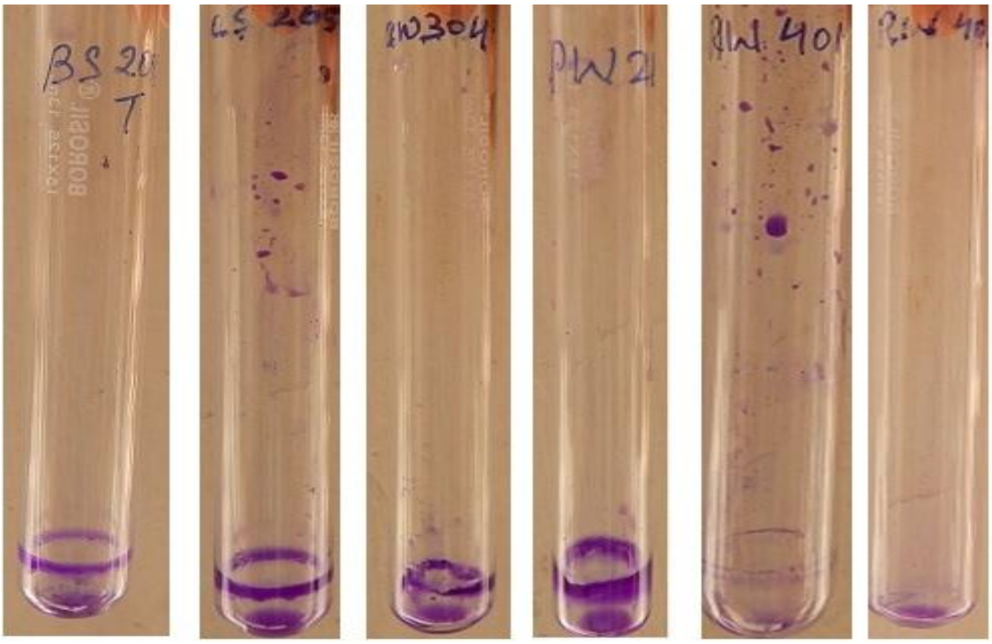
Biofilm screening done by two by classical method explained in methods and material section. For glass tube assay, positive biofilm formation is marked by the emergence of ring in the test tube. The results were listed below, '+' marks denotes better ring formation (Fig 10) and (Table 6).

Table 6. Results of Biofilm assay of bacterial isolates

Strain Number	**Biofilm forming ability**	**Strain Number**	**Biofilm forming ability**
BW03	++	RW 401	+
CS605	-	BS 202T	+++
CW 603	+++	CW 304	+++
GW 502	+	RW 406	+
RW 402	+++	CW 503	+++
PW04	-	RW 203	-
PW401	-	PW 702	++
GW 702	-	PW 216	+++
PW02	-	CS 205	+++
CW 102	+	CS 09	-



RW 402 **BW 03** **CW 603** **GW 502** **CW 102**



BS202T **CS 205** **CW 304** **PW 216** **RW 401** **PW 702**

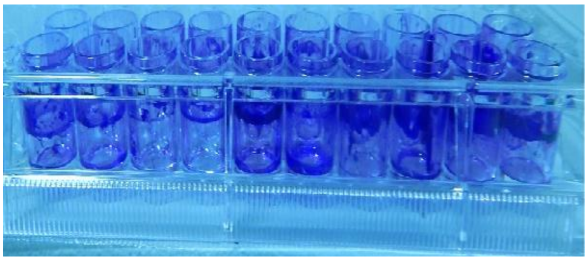


Fig. 10. Result showing the biofilm of bacterial isolates by glass tube assay and micro-titre plate

5.5 *merA* amplification

Bacterial isolates those have non *mer* mediated gene in their genome have shown no band during gel run. In lane no 8 and 9 a band is seen, were in lane no 8, PW-702 showing a presence of *merA* gene in their genome at around 480 bp, were as in lane no 9, PW-216 showing a non specific band at 300 bp. However, in rest of the lanes no band seen, that implies no *merA* gene in those isolates (Fig 11).

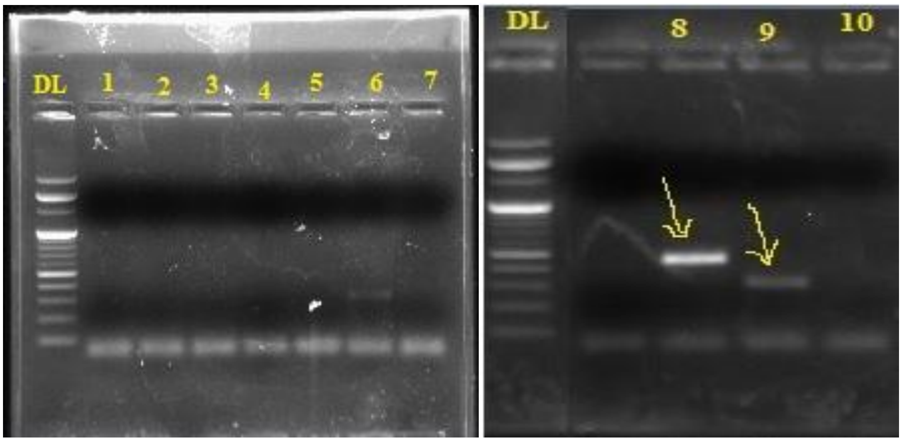


Fig 11. Gel photograph showing *merA* amplification in the isolates: DL implies the DNA ladder, here DL is of 1000 bp. Lane 1-RW-406; Lane 2- RW-203; Lane 3- 401; Lane 4- CS-205; Lane 5- RW-101; Lane 6- CW-304; Lane 7-CW-503; Lane 8- PW-702; Lane 9- PW-216; Lane 10- CS-09

5.6 Extraction of EPS

Extraction of EPS was done both in the presence and absence of mercury, further taken for the FTIR analysis. Only one strain PW-702 showing positive result for *merA* amplification, so it was taken for examine the expression profile analysis using RT-PCR.

5.7 FTIR Analysis of the extracted EPS at different time interval

FTIR analysis of EPS with Hg and pristine EPS was done to examine the presence of functional group and a specific wave number and to interpret the binding of Hg to EPS. FTIR analysis of pristine EPS at different time interval was shown below (Fig 12) and (Table 7).

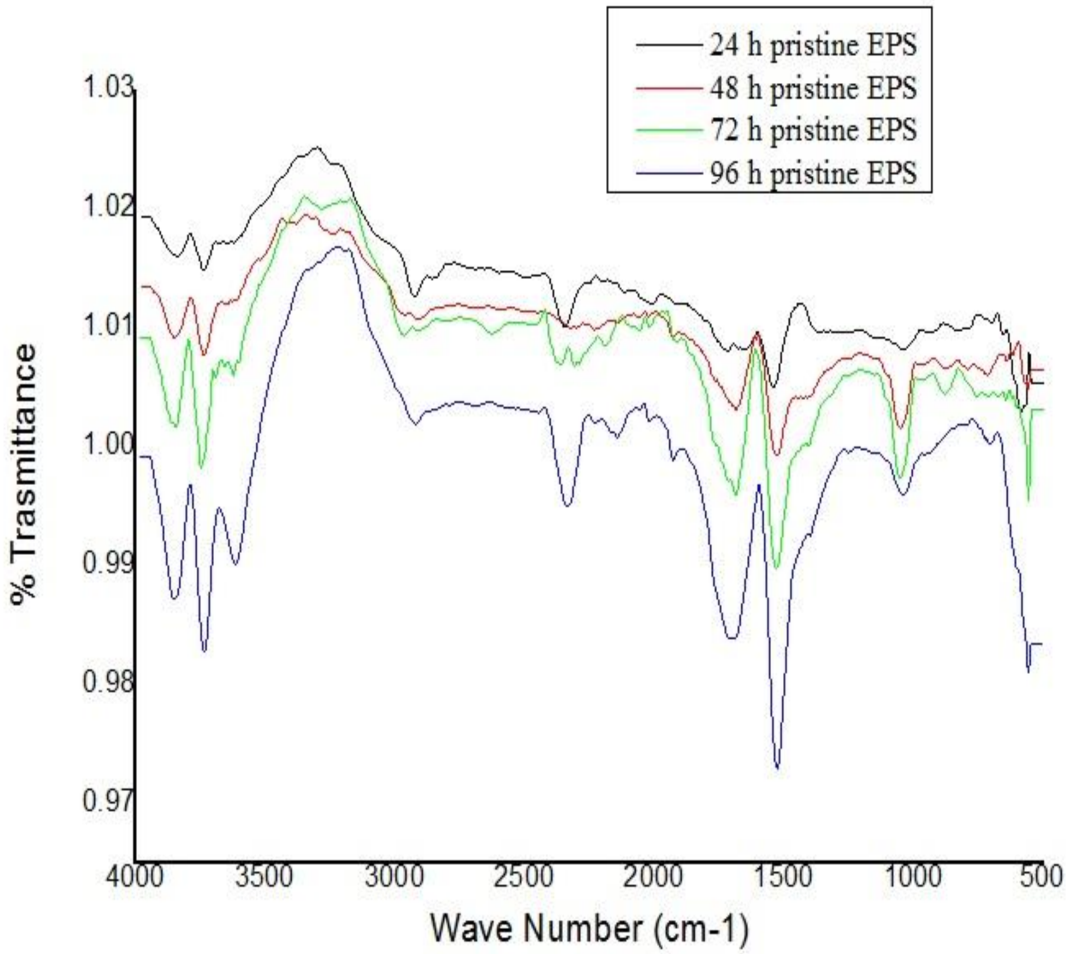


Fig 12. FTIR analysis of pristine EPS at different time interval

Table 7. FTIR analysis of pristine EPS at different time interval

Functional Group Stretch	Wave Number at different Time interval (cm^{-1})			
	24 h	**48 h**	**72 h**	**96 h**
SH Stretch	2395	2338	2338	2338
C-S Stretch	695	708	708	708
C=S Stretch	1239	1251	1251	1251
S-S Stretch	700	682	700	700
S=O Stretch	1156	1156	1150	1156

5.8 Expression of *merA* gene

Expression of *merA* gene was examined using RT PCR and result shows a variable gene expression at different time interval. Gradually increase in expression was observed and the most impressive expression is seen in 96 h and the least expression is in 24 h (Fig 14).

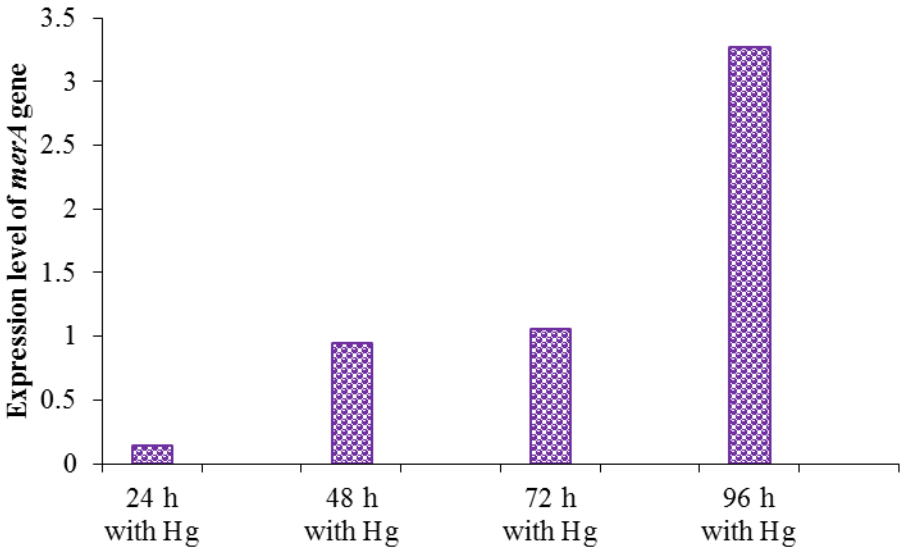


Fig 13. Graphical representstion of expression profile of *merA* gene

6. Discussion

Mercury always been a matter for concerned because of its frequent use in the globalised industries and because of its life threatening effects. An effect of mercury is not hidden from anyone now a day, proper remedy should be taken for abolishing its toxic effect. As we all know, microbes which were unaided to human eyes plays a vital role for the environmental concerned, so we tried to employ a marine bacteria which can help it out with the remediation process. Our study is basically concentrated upon the remediation of mercury by detoxifying its noxious form using mercury resistant marine bacteria. Isolates were taken from many biological coastal sites; especially samples were collected from coastal side of Odisha. During collection it's obvious that many undesirable samples were also collected so screening of samples is very important. Initially we examine the samples for their tolerance toward the different concentration of Hg, so we employ MIC (Minimal inhibitory concentration) determination method (CLSI, 2006) by which we can estimate the minimum concentration of their tolerance. It was seen that the samples were shown a minimum tolerance to variable range of Hg concentration around 3 to 50 ppm. We were also seeking for a bacterial strain which possess biofilm forming ability within it, so that the further idea related to our study will carry out in a proper direction, for that we use glass tube assay and micro-titre assay (Jain et al., 2014), there we seen that some strains (CW-603, PW-702, RW-402, BS-202T, CW-304, CW-503, PW-216 and CS205) showing a very good attachment by forming a biofilm. As we got some biofilm forming strain now we tried to track down the strain's genetic quality of having a *merA* gene within it so that the detoxifying process can initiated after the accumulation of Hg by bacteria, here we got one strain (PW-702) with *merA* positive when we did a amplification of *merA* and visualize the amplification using Agarose gel electrophoresis and a emergence of band at 480 bp confirms the presence of such novel gene. Here onward our study is narrow down to a single strain PW-702 as it shows all the required criteria of our study, eventually it's a prime task to know the nature of this isolate (PW-702) and by 16S rRNA gene sequencing the isolate was identified to be *Bacillus* sp. Next step is FTIR analysis from which we can able to look forward the binding of Hg to EPS at specific wave number which will provide the clue of Hg accumulation by the isolate.

Our main interest is to know the thiol functional stretches because the accumulated Hg initially binds to the cysteine residue and *merP* and *merT* transport the Hg to periplasm and further *merA* perform the activity by detoxifying Hg by NADP reductase mechanism. For FTIR analysis EPS is a required, we extracted EPS at different time interval at variable Hg stress. Accumulation of Hg shown a left shift by generating a peak to a specific wave number and these proves the accumulation and also provide the sufficient evidence that a PW-702 has an ability to accumulate Hg and can further degrade its noxious effect. Finally we went for examine the *merA* expression by using a RT PCR technique to see the expression profile of *merA* were an impressive expression is seen in 96 h.

7. Conclusion

Among the 20 isolates some were standing tall by showing a tolerance of Hg at different concentration and others by showing the potential of biofilm forming ability. However the isolate which taken the importance is only *Bacillus* sp. PW-702 because of possessing the genetic quality of having *merA* gene within their genome. The task not ends here, our major concerned is to know whether the isolate accumulate the Hg or not so during FTIR analysis it provide the best result by providing a desire peak at specific wave number which proves that *Bacillus* sp. PW-702 accumulating the Hg and has the potential to degrade the noxious effect of Hg during further process govern by respected gene, even during expression study of *merA* using RT PCR the same evidence is accounted for *Bacillus* sp. PW-702.

A class of Mercury resistant marine bacteria (MRMB) those were isolated from contaminated environments is exceptionally potential to minimize mercury from contaminated realm. So, it is propose that mercury elimination ability of this bacterium should be examined. Moreover this isolate *Bacillus* sp. PW-702 can be genetically engineered to acquire better results in removal of mercury. However, before using the strain as an efficient biotechnological tool for mercury detoxification, try to get to the bottom of this strain. Further investigation needs to be carried out in laboratory scale and in-situ metal reduction potential of the genus has to be assessed.

7.1 Future perspective

The following conclusions can be extracted from the present investigation:

i. Chilika, Bhitarakanika, Gopalpur, Paradeep and Rushikulya are mercury polluted sites in the Odisha coast.

ii. Bacterial community play a vital role in bioremediation by reducing the toxic form of mercury to non toxic form either by converting toxic form to non toxic form or by accumulating mercury within them.

iii. One mercury resistant bacteria were isolated that helped in detoxification of mercury in the environment.

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Appendix

A. Media:

The media used and their compositions are given below:

Table 1: Details of media used and their composition

1. Sea Water Nutrient Agar (SWNA):

Components Quantity (Gram's/Litre)

Peptone 5.0 g

Yeast Extract 3.0 g

NaCl 15 g

Agar Powder 15 g

Milli Q 1000 ml

pH (at 37^{0}C) 7.5±0.1

2. Mueller Hinton Broth (MHB):

Components Quantity (Grams/Litre)

Beef infusion solids 4.0

Starch 1.5

Casein hydrolysate 17.5

pH (at 37^{0}C) 7.4±0.2

3. Luria Bertani Media:

Components Quantity

Tryptone 2.00

NaCl 1.00

Yeast Extract 0.5%

pH (at 25°C) 7.0

B. Stains:

Bacterial isolates were stained by using Gram's staining methods:

TABLE 2: COMPOSITION OF GRAM'S STAIN:

Ingredients Uses

Crystal violet Primary Staining Agent

Safranin Secondary Staining Agent

Lugol's Iodine Mordant

Acetone Decolourising Agent

i) $HgCl_2$ Solution:

$HgCl_2$ 15 g

Conc. HCl 2.5 g